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In two separate reports, breast cancer cells were shown to express functionally active chemokine receptors which may promote metastasis, and an anti-human CXCR4 chemokine receptor monoclonal antibody was found to reduce the level of lung metastasis by 61-68 percent. Based on these findings supporting the role for chemokine ligand-receptor interactions in promoting metastasis of breast cancer, we develop small molecule antagonists to CXCR4. This was accomplished by screening in a competitive assay synthetic combinatorial libraries (SCLs) made up of D-amino acid peptides for their ability to antagonize CXCR4 receptor function using HeLa cells and PBMC cells (used as standard), and breast cancer cells (MDA-MB-231, known to express CXCR4), and a monoclonal antibody anti-CXCR4 known to block chemotaxis induced by CXCL12 (formerly known as SDF1-α). The SCL approach, particularly when generated in a positional scanning (PS) format, allows the direct identification of the key residue(s) of active peptide sequence(s) from the library screening. Following the screening of a library, candidate sequences were synthesized and their inhibitory activity on the binding of anti-CXCR4 antibody was evaluated as well as their ability to abrogate the migratory response of cells induced by SDF-1α.

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INTRODUCTION

Breast cancer cells were recently reported to express functionally active chemokines which may promote metastasis (1). Specifically, chemokines (e.g., SDF-1α) and their receptors (e.g., CCR7 and CXCR4) are now believed to play a critical role in motility, homing, and proliferation of cancer cells at specific metastatic sites. For instance, the signaling pathways activated upon interaction of CXCR4 with SDF-1α play a role in the survival and proliferation of cancer cells once they are localized in a specific organ. These *in vitro* and *in vivo* inhibition studies make CXCR4 a potential target for preventing the conversion of premalignant to invasive breast cancer. The overall aim of this proposal was therefore to develop small molecule chemokine antagonists that can be used as simple model systems to further understand the role of chemokine ligand-receptor interactions in breast cancer evolution, as well as lead compounds for potential treatment against metastatic breast tumors. This was accomplished by using a synthetic combinatorial library (SCL) approach to identify D-amino acid hexa- and decapeptides that block the anti-CXCR4 antibody binding to CXCR4-expressing cells. D-peptides, by their nature, are much more stable to proteolysis and immune clearance and are attractive alternate drug candidates to natural L-peptides.

RESEARCH ACCOMPLISHMENTS

Task 1: To determine the experimental conditions for an optimal detection of CXCR4 expression by flow cytometry

The detection of CXCR4 recognition by Cy-chrome labeled anti-CXCR4 mAb 12G5 (Pharmingen, San Diego, CA) was evaluated by flow cytometry for two breast cancer cell lines, MDA-MB-231, known to express CXCR4 (2) and MDA-MB-468, a CXCR4-deficient cell line (1) used as negative control, as well as for HeLa cell line and peripheral blood mononuclear cells (PBMCs) from healthy volunteers, used as positive controls. The corresponding isotype IgG2a was used as a negative control to substract non-specific binding. A 30 min incubation at room temperature of $2x10^6$ cells with 5 µl mAb resulted in 8% and 4% specific recognition for MDA-MB-231 and MDA-MB-468 cells, respectively, and 80 to 85% specific recognition for HeLa and PBMCs. The low recognition of the MDA cells observed in our flow cytometry measurements agrees with a recent finding reported by Helbig et al. (3) that less than 10% of MDA-MB-231 cells grown in culture express CXCR4 on their surface. A prerequisite to identify individual active compounds from mixture-based combinatorial libraries is a significant signal to noise ratio. Due to the low expression of CXCR4 on MDA cells, we selected to use PBMCs as standard cells to develop CXCR4 antagonists from libraries and evaluate the antagonistic activity of the best candidates with breast cancer cells.

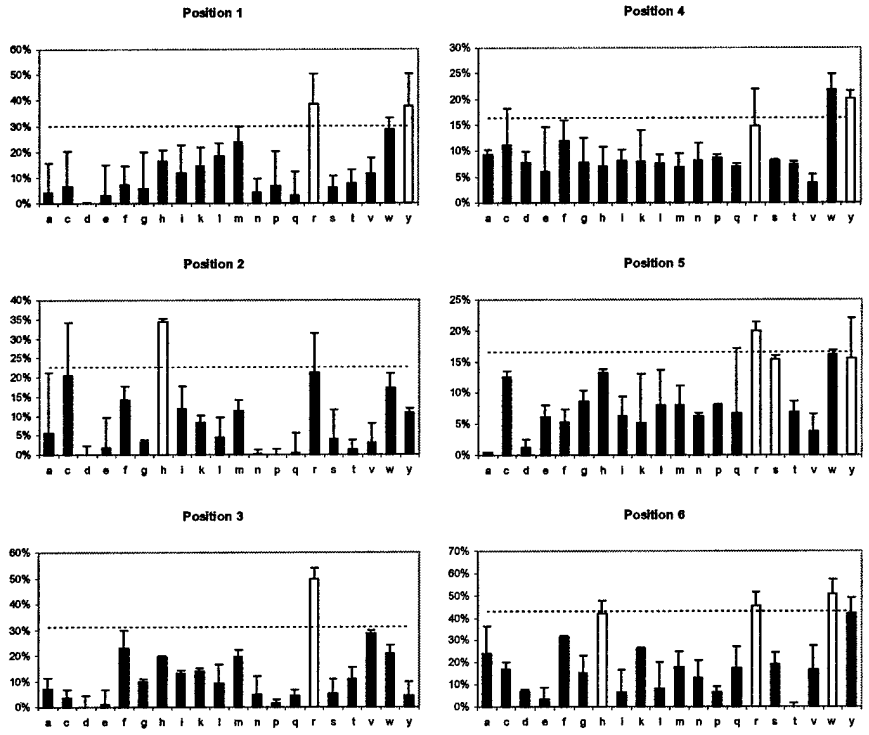
Task 2: To screen two peptide synthetic combinatorial libraries for their ability to antagonize CXCR4 receptor function

The libraries used in these studies are mixture-based SCLs and represent collections of peptides that are screened as mixtures of soluble compounds [i.e., not attached to solid supports (4)]. They were generated in a positional scanning (PS) format, which allows the determination of key residues at all diversity positions in a single screening assay (5). Thus, a PS-SCL is composed of sublibraries, each addressing a position in a peptide sequence. The data

Table 1. Hexapeptide PS-SCL description

- 6 diversity positions - 1 defined position - 6 sublibraries	- 20 D-amino acids at each defined position (o) - 19 D-amino acids at each mixture position (x - no D-cysteine)
Ac-oxxxxx-NH_2 Ac-xoxxxx-NH_2 Ac-xxoxxx-NH_2 Ac-xxxoxx-NH_2 Ac-xxxxox-NH_2	- 20 mixtures per sublibrary - 6 x 20 = 120 mixtures to be tested - $19^5 \sim 2.5 \times 10^6$ peptides per mixture - $20 \times 19^5 \sim 5 \times 10^7$ peptides in entire library

Figure 1. Inhibition of CXCR4 recognition by each mixture making up the D-amino acid hexapeptide library. Each graph represents the inhibitory activity of a given sublibrary. Each bar represents the percent inhibition of a mixture defined with the amino acid listed on the x-axis at 100 μg/ml. The horizontal line represents the average percent inhibition plus 1.5 standard deviation value for all mixtures present in a given sublibrary and is used to differentiate those mixtures having significant activity relative to the others. The hollow bars represent the mixtures that were selected to carry out the deconvolution process.



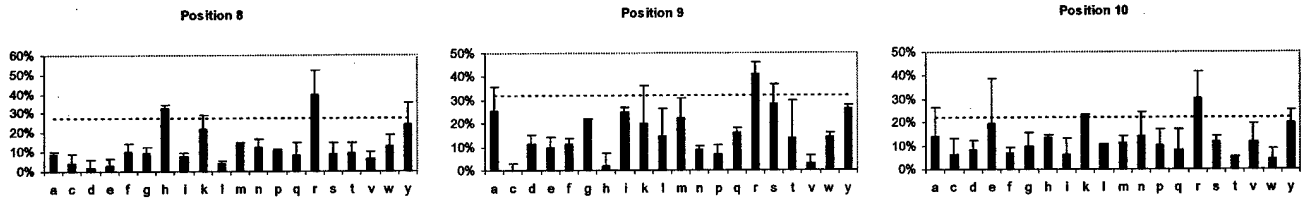
derived from each sublibrary yield information about the key residue(s) for each position. If the key residue(s) at each position represent the same active peptide(s) present in the corresponding mixtures, then combinations of these residues lead to active individual peptides.

The first library to be screened was made up of D-amino acid hexapeptides as described in Table 1. Thus, each mixture was defined by a single D-amino acid ("o" position) while the five other positions ("x" position) were a mixture of 19 D-amino acids; D-cysteine was omitted to avoid polymerization. For example, the first mixture was defined with alanine at position 1 (Ac-axxxxx-NH_2), i.e. contained all possible hexapeptide sequences having an alanine at position 1, and mixture number 120 was defined with tyrosine at position 6 (Ac-xxxxxy-NH_2), i.e., contains all possible hexapeptide sequences having a tyrosine at position 6. Therefore, each individual hexapeptide was present in 6 separate mixtures, each one differing from the position of the fixed amino acid. All 120 mixtures were mixed with PBMCs and the recognition of cell-surface coreceptors by a monoclonal antibody specific for CXCR4 was monitored by FACS as follows. $6x10^5$ cells/ml PBMCs that were pre-activated with human recombinant IL-2 (Pharmingen, San Diego, CA) for 8-12 days were incubated with peptide mixtures for 30 min at 37°C under 5% CO2 atmosphere in FACS solution (PBS, 0.1%BSA, 0.1% Na azide) followed by a 30 min incubation at room temperature with 10μl of anti-CXCR4 mAb 12G5. The corresponding isotype IgG2a was used in all experiments. The cells were washed with 2 ml of FACS solution, resuspended in 0.5 ml of FACS solution, and analyzed using a FACScallibur (Becton Dickinson). The specific fluorescence corresponding to the cell surface molecule was calculated as median of all positive events substracting the fluorescence of the isotype control.

As shown in Figure 1, a limited number of mixtures exhibited antagonistic activity, indicating the presence of a limited number of active sequences within the library. The next step following the screening of a PS-SCL is to synthesize all sequences corresponding to all possible combinations of the amino acids defining the most active mixtures from each sublibary. We selected mixtures that inhibit CXCR4 recognition at a percentage above the average plus 1.5 standard deviation value within each

sublibrary in a manner that the amino acids defining those mixtures were of different chemical character (hollow bars in Fig. 1). For example, only one of the two mixtures defined with tyrosine or tryptophan were selected at positions 1, 4, 5, and 6. This lead to the generation of 36 individual peptides (2x1x1x2x3x3 = 36). In addition, a number of arginine/tyrosine L- and D-amino acid hexapeptides that were generated for an unrelated study were selected for further evaluation as described below. Similarly to known CXCR4 antagonists, the D-hexapeptides to be synthesized and/or tested have a cationic character. The site of recognition in the extracellular domain of CXCR4 that can be blocked with the mAb 12G5 includes an anionic region (6), which could interact with cationic peptides.

Figure 2. Inhibition of CXCR4 recognition by each mixture making up the D-amino acid decapeptide library.



Since the 9-mer poly-D-arginine ALX 40-4C (7) and 10-mer D-peptides derived from SDF-1α (8) were shown to inhibit HIV-1 entry by targeting CXCR4, we also initiated the screening of a D-amino acid decapeptide PS-SCL. This library was built in a manner similar to the hexapeptide library described above (Ac-oxxxxxxxxx-NH_2 to Ac-xxxxxxxxxo-NH_2) and consisted of 200 separate mixtures arranged in 10 sublibraries. Each mixture contained 3.2 x 10^{11} (19^9) different peptides in approximately equimolar concentration, and the entire decamer library contained a total of 6.5 x 10^{12} (20 x 19^9) different individual decapeptides. Three sublibraries were tested so far in a manner similar to the hexapeptide SCL (Fig. 2). As for the hexapeptide library, the most active mixtures are mostly defined with basic residues (arginine or lysine) or tyrosine. While the screening of the library will be completed in the near future, a number of arginine/tyrosine D-amino acid decapeptides that were generated for an unrelated study were selected for further evaluation as described below.

Task 3: To synthesize candidate peptide sequences

All individual peptides were synthesized by simultaneous solid-phase technology using standard t-Boc chemistry and p-methylbenzydrylamine resin (0.81meq/g, 100-200 mesh), which was contained within a sealed polypropylene mesh packet (9). Final cleavage and deprotection steps were carried out with liquid hydrogen fluoride (HF) using a "low-high" HF cleavage protocol (10,11). The identity and purity of the peptides were analyzed by mass spectral analysis interfaced with a liquid chromatography system (Finnigan LCQ) and analytical reversed phase high performance liquid chromatography (RP HPLC) using a Beckman System Gold HPLC. The peptides were purified by preparative RP HPLC using a Waters Milliprep 300 preparative HPLC with a Foxy fraction collector.

Task 4: To determine the antagonistic activity of the candidate peptides

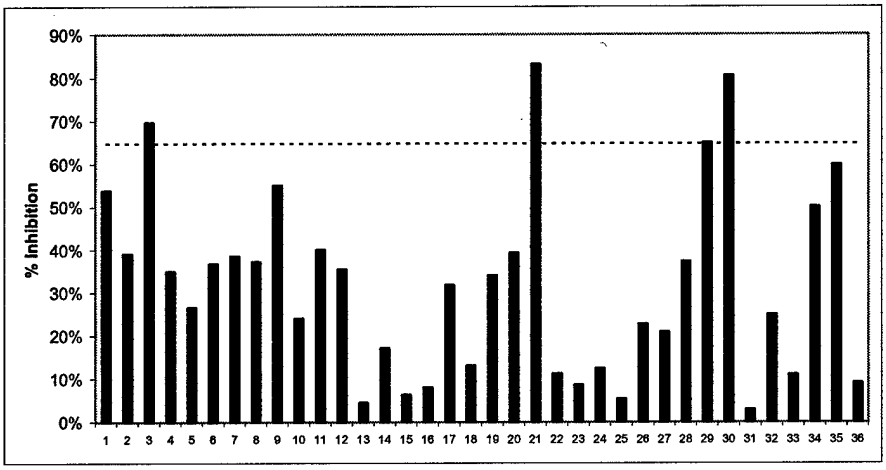
Table 2. Active hexapeptides toward PBMCs

	% Inhibition at 1,000 ng/ml	100 ng/ml	10 ng/ml
Ac- R R W W R R -NH_2	81%	65%	18%
Ac- R R W W C R -NH_2	76%	16%	--
Ac- r r w w c r -NH_2	75%	12%	--
R R Y W W R -NH_2	63%	26%	--
Ac- R R K W R F -NH_2	62%	19%	--
Ac- R R R R R R -NH_2	58%	6%	--
Ac- R R W W R F -NH_2	53%	37%	--

In a first experiment, the 36 D-amino acid hexapeptides derived from the library were assayed for inhibition of CXCR4 recognition using PBMCs. As shown in Figure 3, the most active peptides exhibit 65 to 83% CXCR4 recognition at 1μg/ml.

In parallel, 14 L- or D-amino acid hexapeptides derived from unrelated studies were similarly tested. The most active peptides are

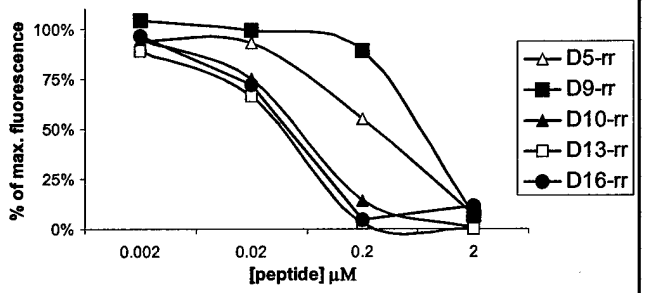
Figure 3. Inhibition of CXCR4 recognition in PBMCs by individual D-hexapeptides



#		% Inhibition
21	Ac- y h r w r w -NH_2	83%
30	Ac- y h r y r w -NH_2	81%
3	Ac- r h r w r w -NH_2	70%
29	Ac- y h r y r r -NH_2	65%

listed in Table 2. The most active L-amino acid hexapeptides inhibited CXCR4 recognition with an EC_{50} value of 37 ng/ml.

Figure 4. Inhibition of CXCR4 recognition in PBMCs by D-decapeptides



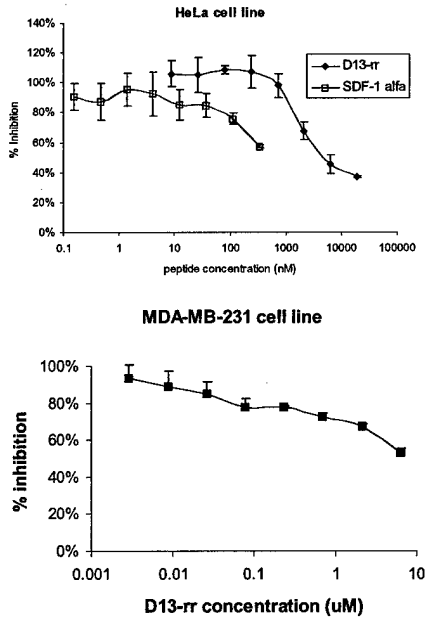
Name	Sequence
D5-rr	Ac-rrwrrriyrr-NH_2
D9-rr	Ac-rrwrrriyyr-NH_2
D10-rr	Ac-sfmyrrwrrr-NH_2
D13-rr	Ac-rrmyrriyrr-NH_2
D16-rr	Ac-rrmyryhrui-NH_2

EC_{50} at 29 nM levels (Fig. 4). D10-rr and D16-rr were similarly effective while D5-rr exhibited a 10-fold lower activity (EC_{50} values of 233 nM – Fig. 4).

An alternative assay to flow cytometry was investigated to determine whether the identified peptides inhibit CXCR4 recognition by mAB 12G5. An ELISA

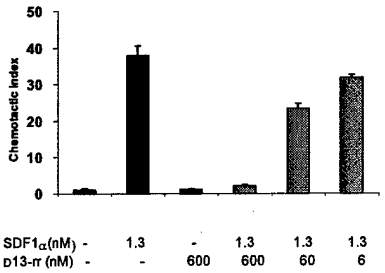
Similarly, a set of D-amino acid decapeptides were assayed for inhibition of CXCR4 recognition. The most active peptide (D13-rr) exhibited antagonistic activity with

Figure 5. ELISA for determination of inhibition of CXCR4 recognition



assay was set up in which the peptides were preincubated with the cells overnight at 4°C, followed by 1 hr incubation at 37°C with mAB 12G5 and the inhibition was detected using europium-labeled streptavidin. As a positive control, HeLa cells were first used to ensure detection of antagonistic activities. In this assay, D13-rr inhibited CXCR4 recognition in HeLa cells with EC_{50} at 1.5 μM levels, i.e., 10-fold lower than the natural ligand SDF-1α (Fig. 5). Using the same experimental conditions (25,000 cells per well), a slight inhibition of CXCR4 recognition by mAb 12G5 could also been observed for D13-rr when testing MDA-MB-231 cells (Fig. 5). Optimization studies are on going to determine the experimental conditions leading to an increased detection level of inhibition.

Figure 6. Inhibition of chemotactic activity of SDF-1α on CCRF-CEM cells in the presence of D13-rr.



We also initiated studies to evaluate if the peptide candidates have antagonistic effects on the chemotaxis induced by SDF-1α. Since CCRF-CEM cells are non-adherant CXCR4 expressing cells known to migrate, the migration of CCRF-CEM cells in the presence of D13-rr was evaluated using a Transwell system with a membrane of 5μm. 600 nM D13-rr could completely block the cell migration induced by 1.3 nM SDF-1α, while partial inhibition was observed with 60 nM D13-rr (Fig. 6). These results support that the peptide interacts with CXCR4 to block the binding site of SDF-1α or to induce a conformational change of the binding site of SDF-1α in CXCR4.

The remaining of the project will be directed toward a detailed evaluation of the antagonistic activity of the identified peptides using breast cancer cells. Emphasis will be given to the optimization of the experimental conditions for ELISA and chemotatic assay.

KEY RESEARCH ACCOMPLISHMENTS

- Identify novel short peptides that block the recognition of CXCR4 by anti-CXCR4 mAb 12G5.
- The peptide candidates block the chemotaxis of CXCR4 expressing cells induced by the natural ligand SDF-1α

REPORTABLE OUTCOMES

None

CONCLUSION

Using a combinatorial library approach, we have identified cationic D-amino acid hexapeptides and decapeptides that block the anti-CXCR4 antibody binding to CXCR4-expressing cells. The unique nature of these D-peptide inhibitors (i.e., length and nature of their sequences), as well as the importance of CXCR4 on breast cancer metastasis make these peptides useful tools to further explore the elements responsible for their inhibitory activity in a structure-activity relationship (SAR) study. Such study would provide important information on how small peptides may affect SDF-1α binding to CXCR4, and further our understanding on the role of SDF-1α/CXCR4 interactions in the spread of breast cancer cells.

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